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Mini-Symposium

Redefining Noradrenergic Neuromodulation of Behavior: Impacts of a Modular Locus Coeruleus Architecture

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The locus coeruleus (LC) is a seemingly singular and compact neuromodulatory nucleus that is a prominent component of disparate theories of brain function due to its broad noradrenergic projections throughout the CNS. As a diffuse neuromodulatory system, noradrenaline affects learning and decision making, control of sleep and wakefulness, sensory salience including pain, and the physiology of correlated forebrain activity (ensembles and networks) and brain hemodynamic responses. However, our understanding of the LC is undergoing a dramatic shift due to the application of state-of-the-art methods that reveal a nucleus of many modules that provide targeted neuromodulation. Here, we review the evidence supporting a modular LC based on multiple levels of observation (developmental, genetic, molecular, anatomical, and neurophysiological). We suggest that the concept of the LC as a singular nucleus and, alongside it, the role of the LC in diverse theories of brain function must be reconsidered.

Key words: **locus coeruleus; stress; pain; anxiety; executive function; development**

Introduction

The locus coeruleus (LC), a brainstem pontine nucleus of noradrenergic neurons, was identified in the human brain >200 years ago (Fig. 1). Writing in the first journal of German physiology (Reil, 1809; p 511, second paragraph), Johann Christian Reil described his observation: "The anterior extremity of the anterior shank [the *Pedunculus cerebellaris superior*]. . . forms, together with the anterior Marksegel [the *Velum medullare superior*], the roof of the fourth ventricle. In the angle at which the anterior shank comes together with the adjoining area, a stripe of black substance shimmers through, only covered by the epithelium." Reil is referring to the epithelium covering the floor of the fourth ventricle, where the LC is located. He continues: "Only in two places in the brain one finds black substance, here, as well as on the shanks before the bridge [the *pons*]. . . . " (translation by Prof. Almut Schüz at the Max Planck Institute for Biological Cybernetics in Tübingen, Germany; italics added with modern anatomical names). The location of the second area he refers to is consistent with the location of the substantia nigra. These black areas were visible in the human brain macroscopically without staining or microscopy. A few years later, the physicians and brothers Joseph and Karl Wenzel made a similar observation and, communicating their observations in Latin, thus described the *Loci caerulei*, that is, "blue spots" on either side of the brainstem that are now referred to in the singular as the LC (Wenzel and Wenzel, 1812). The original illustrations are shown in Figure 1. The black shimmering substance observed in this incipient research was later discovered to be neuromelanin, a pigment that is thought to be a result of dopamine synthesis (as well as noradrenaline since it is produced from dopamine) (Foley and Banter, 1958; Bazelon et al., 1967; Double et al., 2008).

In the 1960s and 1970s, with the advent of a histochemical reaction that caused catecholamines to fluoresce yellow-green (Falck et al., 1962; Dahlstroem and Fuxe, 1964), as well as a fluorescent antibody for the noradrenaline synthesis enzyme (Hartman, 1973), and autoradiographic methods (Jones and Moore,

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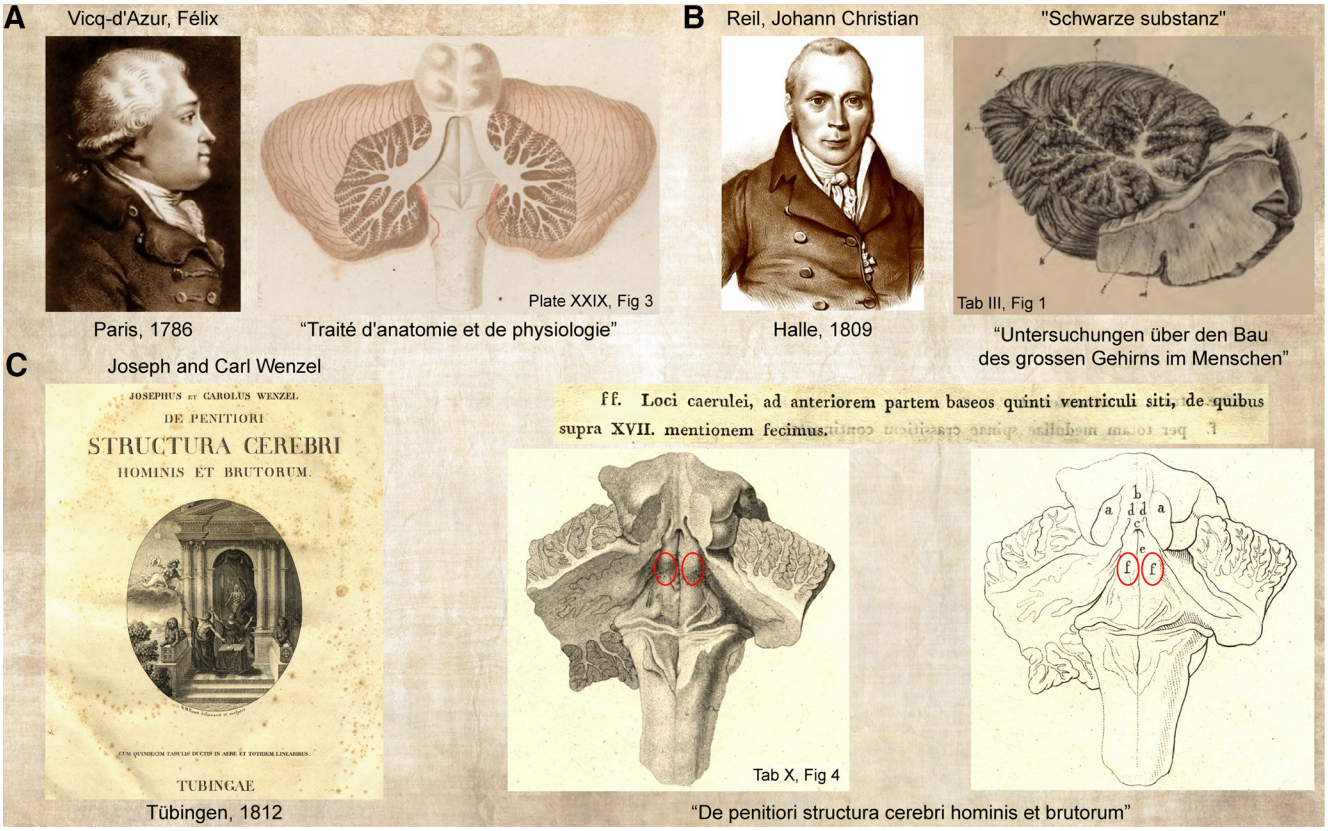

Figure 1. Drawings of the pons by Vicq-d'Azur (1786); Reil (1809), and Wenzel and Wenzel (1812) and identification of a darkly pigmented area, which was named the *Loci caerulei*. ***A***, Vicq-d'Azur produced detailed drawings of the gross anatomy of the human brain, noting a pigmented area, the *locus niger crurum cerebri*, which is consistent with the substantia nigra. ***B***, Reil (1809) reported a "schwarze substanz" (black substance) in two areas consistent with the substantia nigra and the locus coeruleus. ***C***, The Wenzel brothers also reported a pigmented structure on the roof of the pons under the fourth ventricle, naming it the *Loci caerulei* from which the locus coeruleus takes its name. The label "ff" refers to the Loci caerulei in the drawings (found in Table X in the book). We have highlighted this area with red ovals. The structure name is on page 341 and is presented as Figure 4 ("Figura quarta," p 340) in Table 10 ("Tabula decima," p 339). Figure provided by N.K.T. and Stefan Hirschberg.

1977; Jones et al., 1977), these pigmented neurons were shown to be noradrenergic and to project broadly, even "globally," ascending across nearly the entire forebrain and descending, too, into brainstem and spinal cord (Swanson and Hartman, 1975; Grzanna et al., 1977; Fallon et al., 1978; Morrison et al., 1979). This led to the concept of the LC as being part of the central arousal system, preparing the brain for effortful cognitive action. Given its diffuse projections, it came as no surprise, then, that this small brainstem nucleus was involved in myriad brain functions. Subsequent electrophysiology and lesion studies demonstrated activation of the LC in the contexts of wakefulness (Foote et al., 1980; Aston-Jones and Bloom, 1981), the orienting reflex (Foote et al., 1980; Grant et al., 1988; Aston-Jones et al., 1994; Bouret and Sara, 2004), flexible cognition involving shifting attention (Aston-Jones et al., 1994), sensory gating (Waterhouse and Woodward, 1980; Waterhouse et al., 1990, 1998; Devilbiss and Waterhouse, 2004), invigorating of goal-directed activity (Anlezark et al., 1973), analgesia (Hirschberg et al., 2017), pain and stress (Igarashi et al., 1979; Elam et al., 1986; Valentino et al., 1991; Hirata and Aston-Jones, 1994; Mana and Grace, 1997; Sajedianfard et al., 2005; Hickey et al., 2014; McCall et al., 2015), and fear conditioning as well as fear extinction learning (Mueller et al., 2008; Uematsu et al., 2017; Giustino et al., 2019).

A central question that LC research has oft considered is how a broadly projecting nucleus could affect any singular function without affecting them all. For example, LC activation triggers awakening and arousal (Carter et al., 2010; Hayat et al., 2019), which is associated with enhanced sensory discrimination (Aston-Jones et al., 1994; Martins and Froemke, 2015) and lowered sensory neuron response thresholds (Waterhouse et al., 1990, 1998; Manunta and Edeline, 1998, 2004; Bouret and Sara, 2002; Devilbiss and Waterhouse, 2004; Devilbiss et al., 2006; Edeline et al., 2011; Navarra et al., 2013), but this sits at odds with the observation that LC activation is also associated with suppression of nociceptive sensory inputs (analgesia). This apparent paradox is readily demonstrable in attentional analgesia paradigms in humans where LC activity is associated with the interaction between attention (increased visual sensory discrimination) and analgesia (diminished nociceptive percept) (Brooks et al., 2017). One perspective, which has ample support, is that the LC alters global noradrenaline concentration and specific functional consequences are achieved through differences in postsynaptic receptors and regional differences in the spatiotemporal dynamics of noradrenaline reuptake (Berridge and Waterhouse, 2003; Agster et al., 2013; Giustino and Maren, 2018). However, a complementary view is now emerging that suggests that the LC may provide localized neuromodulation via LC neurons that have relatively circumscribed projection targets and synchronous spike timing among only subsets of LC neurons (for review, see Totah et al., 2019). In many ways, this perspective has parallels with the emerging conceptualization of the sympathetic nervous system as having discrete efferent limbs that are organ- or even target-tissue-specific with characteristic patterns of activity (for review, see Jänig, 2006) but that also has the capability to act as a unified

whole (Farmer et al., 2019). Similar parallels may be drawn with the dopaminergic system which, over the past decade, has become parcellated by cell type and by cell-specific afferents and efferents that allow this "diffuse" neuromodulatory system to contribute highly informative signals that govern specific cognitive processes (Lammel et al., 2012; Watabe-Uchida et al., 2012; Beier et al., 2015; Tian et al., 2016).

In this review, we will cover recent findings by the authors as well as others in the field of LC research that elaborates this emerging perspective using techniques that have made longstanding questions tractable for detailed investigation. For example, work in rats has demonstrated that subpopulations of LC neurons differentially project to the PFC and motor cortex (Chandler et al., 2013, 2014). Other work has demonstrated that subpopulations of LC neurons send separate projections to the basolateral amygdala (BLA) and infralimbic division of the PFC in rats, which may underlie the role of the LC in seemingly opposing functions of fear extinction and fear conditioning (Uematsu et al., 2017). Similarly, different subpopulations of LC neurons project to the spinal cord versus the PFC to mediate analgesia as opposed to aversion/anxiety, respectively (Li et al., 2016; Hirschberg et al., 2017). Of course, the capacity for targeted neuromodulation depends not only on projection specificity, but also on the degree to which spiking is desynchronized at the population level and synchronized only among subsets of LC neurons. High-channel density electrophysiology has demonstrated such ensemble firing patterns in the rat LC (Totah et al., 2018). Much of the recent progress is attributable to the innovative use of new methodological tools, such as novel retrograde tracing methods, functional manipulations of specific cell populations, and genetic fate mapping. We will review how these methods have advanced our understanding of the LC and its potential to regulate specific functions.

Specific efferent pathways provide targeted neuromodulation of specific functions

There are several accounts of a correlation between the morphology of individual LC neurons, their location within the nucleus, and their terminal projection fields. Mason and Fibiger (1979) first described an efferent topography of the nucleus in rats by injecting the retrograde tracer HRP into various structures throughout the neuraxis. They found that injections into hippocampus or septal nuclei consistently filled cells located in the dorsal, but not ventral, portion of the core of the LC nucleus, whereas injections into motor-related structures, such as caudate-putamen and cerebellum, labeled both ventral and dorsal portions. Injections into thalamus produced labeling in the posterior pole but not in more rostral portions, whereas hypothalamic injections labeled cells in the anterior pole. On the other hand, injections into amygdala and cortical structures, frontal regions in particular, produced labeling of neurons scattered throughout all three axes of the compact core of the nucleus (Mason and Fibiger, 1979; Loughlin et al., 1986a). These findings were confirmed and explored further by both Satoh et al. (1977) and Loughlin et al. (1986b) who showed that these subdivisions of LC, which have disparate efferent targets, also have morphologically distinct cells.

Experiments in which multiple retrograde tracers were injected into different structures in the same brain have yielded conflicting results. By pairing injections of different tracers into cortex and cerebellum, Nagai et al. (1981) showed that a small proportion of labeled LC cells contain axons innervating both structures, while most innervate one area or the other. This was corroborated by others who showed that pairs of injections into cortex and thalamus (Adèr et al., 1980) and cortex and cerebellum (Steindler, 1981) similarly produced small percentages of multilabeled neurons. On the other hand, Loughlin et al. (1982, 1986a) showed that injections of paired fluorescent retrograde tracers into various cortical regions yielded higher proportions of double-labeled LC neurons, especially when injections were made in the same or proximal mediolateral planes. They therefore concluded that single LC cell axons innervate multiple cortical regions as the projection spans mediolaterally across the cortex (Loughlin et al., 1982). An important implication of the work of Loughlin et al. (1982, 1986a) is that LC neurons have been thought to simply tile the cortex with mediolaterally running projections without regard to functional differences between the cortical targets.

More recently, it was shown that LC neurons innervate functionally related structures along ascending somatosensory pathways (Simpson et al., 1997), as well as in nociceptive pathways (Howorth et al., 2009a; Li et al., 2016). It was also shown by several investigators that the projection from LC to cortex is primarily ipsilateral (Jones and Moore, 1977; Mason and Fibiger, 1979; Waterhouse et al., 1983), whereas subcortical and spinal structures receive bilateral input from LC (Simpson et al., 1997; Howorth et al., 2009a). Collectively, these classic studies on LC efferent anatomy suggest that, despite having approximately uniform innervation patterns and function throughout the forebrain, the LC might be able to provide "semiglobal" neuromodulation through a partially targeted neuromodulatory system.

While many classic studies using immunohistochemistry or labeled dyes have characterized LC anatomy, the use of viral vectors and intersectional strategies has revolutionized the ability to trace LC neuroanatomy. This has been powerfully and comprehensively demonstrated through the Allen Mouse Brain Connectivity atlas (www.connectivity.brain-map.org) where the combination of vector-enabled labeling and serial 2-photon tomography have enabled whole brain reconstructions of projections (Oh et al., 2014). The power of this approach is illustrated with respect to the LC (Fig. 2). The selective expression of EGFP in the LC of a TH-Cre mouse line allows the extensive projection tree of the LC to be revealed, forming a global network of fibers across the brain (Fig. 2*B*,*C*). Parcellation of this projection on the basis of projection targets (i.e., olfactory bulb, visual cortex, hypothalamus, medulla) reveals a different perspective with individual fiber tracts targeting these different domains perhaps consistent with modular specialization (Fig. 2*D*). This methodology could, however, equally represent single LC neurons with enormously ramifying axons or, alternatively, that individual LC neurons have distinct projection targets. To address precisely this sort of question, a new viral-genetic neuroanatomical technique called MAPseq has been developed (Kebschull et al., 2016). MAPseq works by transducing a neuron population with a viral library expressing short, random RNA barcodes. Ideally, each neuron receives a single RNA barcode, which is amplified and trafficked to its projections via an axon targeting signal. To determine a neuron's brainwide projection pattern, barcodes are extracted from dissected brain regions and sequenced. MAPseq applied to the LC allowed the projections of these neurons to be resolved at a single-cell level. Overall, individual LC neurons were shown to have highly heterogeneous projection patterns, with some LC neurons projecting to a single brain area (consistent with a modular organization) and others projecting to many (achieving a broadcast signal).

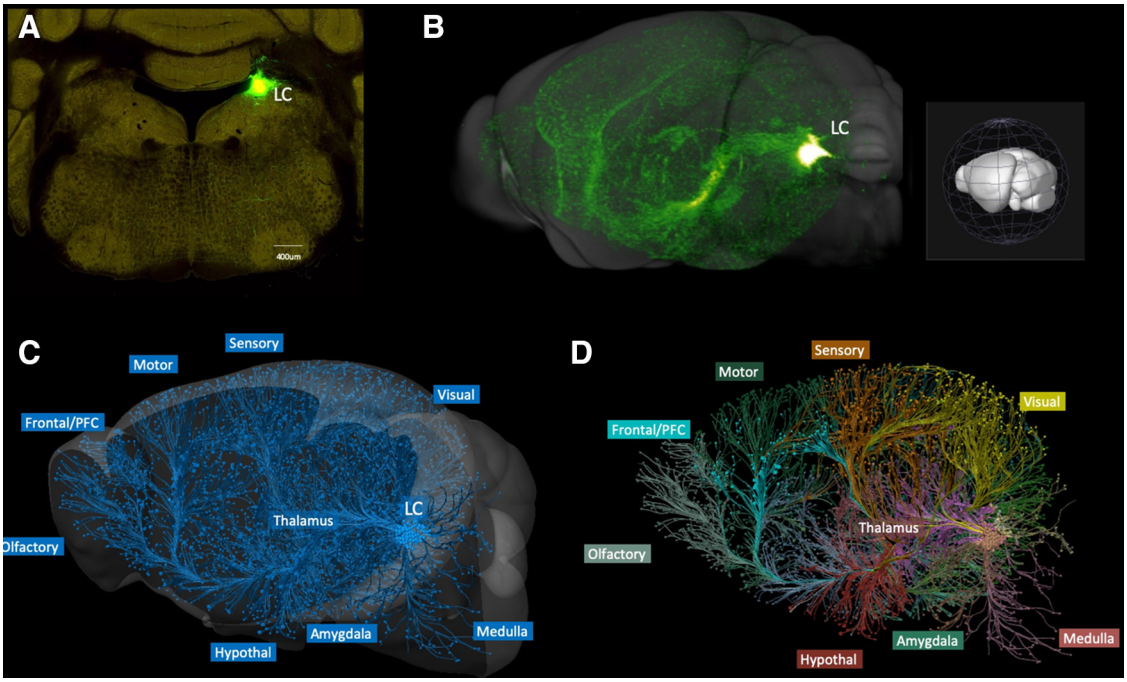

Figure 2. Reconstruction of LC projections suggests a modular architecture. ***A***, Expression of fluorophore in the right LC. A Cre-dependent recombinant adeno-associated virus (AAV) expressing the fluorophore was injected into the mouse LC as an anterograde tracer. The mice were from a Cre-driver line in which Cre recombinase is under control of the promotor for tyrosine hydroxylase (TH), an enzyme expressed by LC neurons as it is required for norepinephrine synthesis (Allen Brain Atlas Connectivity Project Experiment 511971714, TH-Cre_FI172 mouse). ***B***, After 2-photon serial tomography, the LC axonal projections were reconstructed in 3D. ***C***, The distribution of the LC axons is seen to form an extensive network throughout the brain predominantly ipsilateral to the injection (contralateral hemisphere removed). ***D***, Assignation of projection axons by target region reveals an architecture of distinct fiber trajectories consistent with the proposed modular organization. Figure provided by A.E.P.

These advances in viral technologies also make it possible to manipulate the activity of discrete neuron populations with unprecedented specificity *in vivo*. These methods have recently been applied to the LC to test the hypothesis that function-specific neuromodulation is achieved by subpopulations of LC neurons with unique anatomical connectivity in the brain. One approach has been to use the synthetic noradrenergic-neuron-specific promoter, PRS, to selectively express activity-modifying transgenes in LC neurons (Hwang et al., 2005; Lonergan et al., 2005; Howorth et al., 2009a,b; Hickey et al., 2014; Li et al., 2016; Hirschberg et al., 2017; Vazey et al., 2018; Cope et al., 2019; Xiang et al., 2019). Studies using PRS-containing canine adenoviral vectors (CAVs) (Junyent and Kremer, 2015) have been particularly helpful for dissecting the role of specific LC projections by enabling selective optogenetic or chemogenetic activation (Fig. 3). This approach allowed the analgesic effect of ponto-spinal LC neurons to be dissociated from an anxiety/aversive behavior produced by those projecting to the PFC (Hirschberg et al., 2017).

A similar viral strategy was used to uncover important diversity for LC projections related to cognitive behaviors.

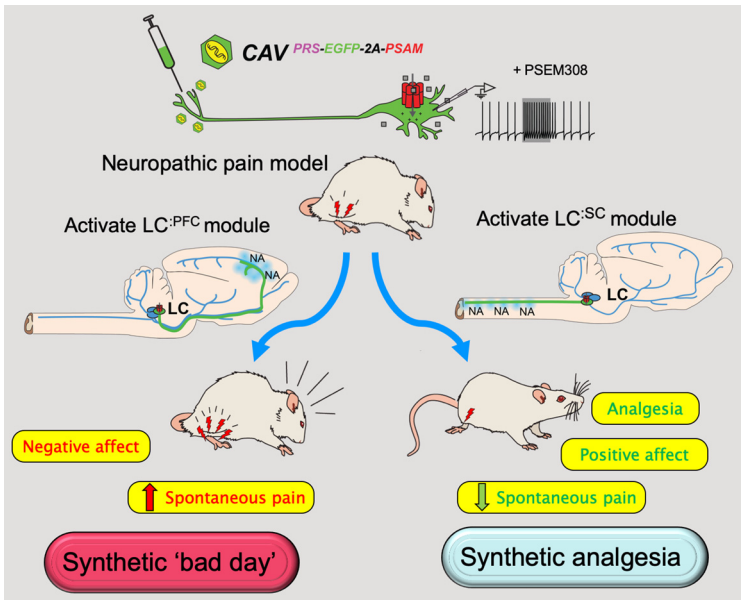

Figure 3. Selective chemogenetic activation of different LC modules bidirectionally modifies the behavioral phenotype in a model of neuropathic pain. A retrograde targeting strategy with a CAV containing the PRS promoter was used by Hirschberg et al. (2017) to selectively express the excitatory chemogenetic actuator (PSAM, modified nicotinic ionophore) in LC modules. This enabled the selective activation (using the agonist PSEM308) of either spinal or PFC-projecting LC neurons during behavioral testing in the tibial nerve transection model of neuropathic pain. Activation of the spinally projecting LC module increased withdrawal thresholds, produced a positive affective bias, and reduced spontaneous pain behavior, consistent with a synthetic analgesic state. In contrast, activation of the PFC projection produced aversion and increased spontaneous pain behavior, which reflects a worsening of the pain phenotype and might be analogous to having a "bad pain day." This analgesic targeting of the spinal LC modules was equally effective preemptively (before nerve injury) and after the nerve injured and the pain phenotype had manifested. Figure provided by A.E.P.

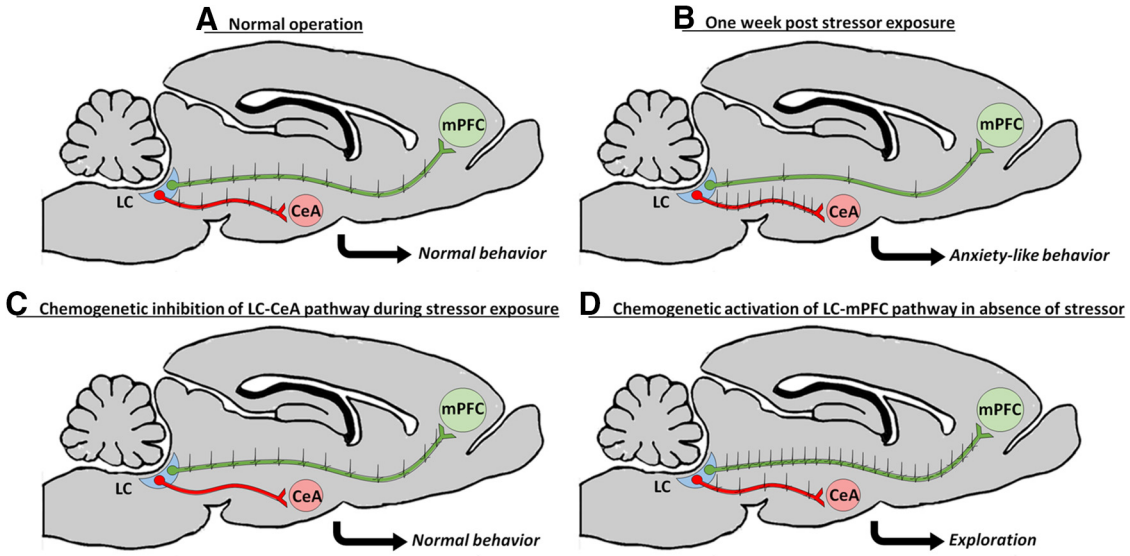

Figure 4. LC projections to PFC and CeA operate in parallel to guide behavior. ***A***, Following normal conditions that do not elicit anxiety-like behavior in male rats, LC cells innervating PFC and CeA show similar levels of spontaneous discharge as assessed by *ex vivo* whole-cell patch-clamp electrophysiology. The level of spontaneous spiking is illustrated by spiking superimposed on the projection fibers. ***B***, One week after a single stressful episode (simultaneous physical restraint and exposure to the predator odor 2,5-dihydro-2,4,5-trimethylthiazoline), rats show increased anxiety-like behavior in the open field. Whole-cell patch-clamp recordings show that LC cells innervating CeA become hyperactive and hyperexcitable, whereas those projecting to PFC show a suppression of activity and excitability 1 week after stressor exposure. ***C***, Injection of CAV-PRS-Cre into either region, followed by an injection of Cre-inducible AAVs to drive expression of designer receptors exclusively activated by designer drugs, (DREADDs) (Roth, 2016) permits manipulation of discrete LC efferent pathways. Inhibition of the LC-CeA pathway during stressor exposure prevents the development of an anxiety-like behavioral phenotype. ***D***, Conversely, activation of the LC-PFC pathway in the absence of a stressor promotes exploration and loss of avoidance of open arms in the elevated plus maze. Figure provided by D.J.C.

Pairing injections of a CAV encoding Cre recombinase (CAV-Cre) at specific LC output sites with injections of Cre-dependent viral vectors into the LC, researchers were able to express optogenetic activators or inhibitors exclusively in LC neurons projecting to the BLA or to the PFC (Uematsu et al., 2017). This allowed the researchers to distinguish the contribution of discrete LC projections to fear and extinction learning. Optogenetic activation of BLA-projecting LC neurons enhanced learning the association between a conditioned stimulus and the unconditioned shock, whereas activating PFC-projecting LC neurons facilitated extinction learning when the shock was no longer administered. Inhibiting these projections at their terminals had the opposite effect: fear learning was reduced, and extinction learning was enhanced. Of significant interest was that inhibiting LC neurons via their terminals had a greater effect on fear-conditioning-related behaviors than indiscriminate inhibition of all LC neurons at their cell bodies, suggesting that manipulating the entire LC may activate opposing frontal-subcortical noradrenergic circuits that could confound behavioral interpretations when activating the LC *en masse*. Similar targeted chemogenetic approaches have demonstrated that the LC-BLA pathway is associated with anxiety and anxiety evoked in rodent models of chronic pain (McCall et al., 2017; Llorca-Torralba et al., 2019). Activation of LC neurons with specific efferent targets versus activation of the entire LC may have differential and even opposite effects on behavior. Such findings illustrate why systemic therapeutic strategies, that augment noradrenaline levels globally within the CNS, are likely to produce a mixed picture of benefits and side effects in patients that are proportionate to the levels of activity in each LC module. This unpredictably increases variability in response between patients and limits the clinical utility of such "globally targeted" noradrenergic-based therapeutics.

One area in which these new viral approaches have had a particular impact is in understanding noradrenergic neuromodulation of PFC-dependent cognitive functions. Prior lesion work suggested that a subpopulation of LC cells innervates subdivisions of the rat PFC to modulate extra-dimensional set-shifting (a type of flexible learning-related behavior) and minimally collateralizes to other PFC subregions involved in other forms of behavioral flexibility (Newman et al., 2008). In line with this idea, early anatomical work had demonstrated that release of noradrenaline from LC efferents is not necessarily consistent throughout the brain: the density of varicosities along noradrenergic fibers is higher in frontal cortex than in motor, somatosensory, and piriform cortices (Agster et al., 2013). New methods have further developed these ideas. Cope et al. (2019) demonstrated that chemogenetic stimulation of LC terminals in PFC facilitates extra-dimensional set-shifting. Moreover, Tervo et al. (2014) have demonstrated that chemogenetic activation of LC neurons projecting to the anterior cingulate cortex promotes stochastic, rather than strategic, behavioral choices.

It has also been suggested that LC neurons projecting to some targets (e.g., the PFC or ACC) differ in their transcriptional and electrophysiological properties from LC neurons projecting to other forebrain targets (e.g., motor cortex) (Chandler et al., 2014). Specifically, cells innervating PFC were found to express higher levels of various genes related to excitability, synaptic transmission, and transmitter synthesis and release than LC cells innervating other cortical regions. In line with these observations, LC-PFC projection cells were found to be more active and excitable and to receive more excitatory synaptic input than other LC cells. Given the difference in excitability between these LC subpopulations, a crucial next step will be to elucidate the specific afferent inputs to these LC subpopulations that may contribute to their activity differences.

Recent unpublished observations by Chandler and colleagues provide further evidence for a modularly organized LC wherein PFC-projecting neurons promote exploration while central

amygdala (CeA) projecting neurons promote anxiety-like behavior (Fig. 4*A*). Additional data suggest that these two subsets of LC neurons undergo opposing physiological adaptations in response to acute stressor exposure to mediate chronic changes in anxiety-like behavior (Fig. 4*B–D*). However, it is important to note that parsing the modularity of LC-mediated anxiety-like behavior may prove to be a difficult task as there is already overlap in the behavioral output mediated by separate efferent projections. Anxiety-like behaviors can be driven by exogenous activation of LC cell bodies (McCall et al., 2015; Sciolino et al., 2016; Li et al., 2018; Zerbi et al., 2019), as well as by activating efferent projections to the BLA, PFC, and superior colliculus (Hirschberg et al., 2017; McCall et al., 2017; Li et al., 2018; Llorca-Torralba et al., 2019). Whether these evoked behaviors are equivalent is difficult to discern when viewed through the lens of a single or small set of related behavioral tasks. Fortunately, the specificity and *in vivo* applicability of viral tools, in combination with microendoscope-based calcium imaging, make it possible to characterize in detail both the activity of LC projections to specific targets and the afferents that drive activity in that subpopulation of LC neurons during multiple behavioral tests.

In summary, current state-of-the-art methods for circuit analysis suggest that at least some populations of LC neurons are more regionally restricted in their axonal collateralization than initially believed, and this can promote specificity in behavioral control. Consequently, the LC has come to be viewed as a modularly organized nucleus capable of segregating several distinct, complex sensory and behavioral functions among subsets of anatomically defined neurons with unique efferent projection fields.

Developmental and genetic characteristics of LC neurons, defining modular architecture?

Using information about efferent projections as a starting point for activating or inhibiting different subpopulations of LC neurons has revealed that the LC is functionally ordered and modular; however, this circuit-based approach is dependent on the use of viral constructs and is therefore typically used in juvenile and adult animals. An alternative strategy for uncovering heterogeneity is based on genetic neuroanatomy, specifically: correlating embryonic gene expression with adult brain structure and function (Joyner and Sudarov, 2012). Unlike the circuit-based approach, genetic neuroanatomy potentially allows reproducible access to subsets of LC neurons across developmental stages (albeit typically restricted to mouse). Genetic neuroanatomy is revealed through an approach termed "genetic fate mapping," which uses cell-type-specific expression of recombinases to switch on a reporter transgene as a lineage tracer (for review, see Jensen and Dymecki, 2014).

For the noradrenergic system, a Cre/loxP and flp/frt dual-recombinase-mediated intersectional genetic fate-mapping strategy was used to subdivide the mature system based on gene expression differences along the anteroposterior axis of the embryonic hindbrain. Populations of noradrenergic neurons were delineated by genes defining unique progenitor domains of the embryonic hindbrain and by subsequent expression of the noradrenergic marker dopamine β-hydroxylase (Robertson et al., 2013). Using this approach, four subsets of neurons were identified, each distinct in their anatomical distribution and efferent projection pattern. In addition, an unexpected projection to the orbital frontal cortex and insular cortex was found arising from outside of the LC, contradicting the dogmatic view that the LC is the lone noradrenergic nucleus projecting to the cortex (Robertson et al., 2013). Although this fruitful analysis provided multiple molecular points of entry to study the noradrenergic system, the analysis revealed limited molecular heterogeneity within the LC. Greater than 99% of LC-NE neurons are derived from the embryonic hindbrain progenitor domain defined by *En1* expression. While this finding has allowed noninvasive and reproducible genetic access to study LC function in isolation from all other central and peripheral noradrenergic neurons (Sciolino et al., 2016; Chen et al., 2019), uncovering heterogeneity within the *En1*-defined LC would require new genetic tools.

Building on the cre/loxP and flp/frt dual-recombinase-based intersectional genetic strategy developed by Awatramani et al. (2003), dre/rox was used to develop a triple-recombinase-responsive indicator allele (Plummer et al., 2015). Using this new indicator allele, the LC, as defined by a history of *En1* and dopamine β-hydroxylase expression, can be further subdivided by a third gene expression domain for experimental study. The circuit approach can be merged with genetic fate mapping by virally delivered recombinase injected into LC targets. Thus, LC modules defined by efferent projections and function can be experimentally subdivided further by developmental or adult gene expression.

This approach was recently used to reveal that developmental gene expression generates differences in LC efferent neuronal circuitry. The triple-recombinase-based fate-mapping approach was used to subdivide the *En1* progenitor domain along the dorsoventral axis, using *Pax7* as a marker of the dorsal alar plate of the neural tube. In the adult brain, LC neurons with a history of *Pax7* expression are intermingled with *Pax7*-negative LC neurons (Plummer et al., 2017). Although these two LC neuronal populations are intermingled throughout the extent of the rostrocaudal and dorsoventral axes of the LC, they differ in their efferent projection profiles, suggesting that they may be functionally distinct. Both populations project to most cortical regions, but whereas *Pax7*-positive LC neurons project to the thalamus, the projection from *Pax7*-negative LC neurons is extremely sparse to virtually absent. While this finding highlights the interaction of development and LC efferents in the formation of LC modules, the ultimate impact of this specific finding on behavioral function is unclear. New tools that bridge developmental genetic neuroanatomy and efferent projections to define and functionally manipulate LC modules promise to increase the resolution at which complex systems like the LC can be functionally dissected.

The emerging data on the genetic profile of LC neurons (Robertson et al., 2013; Chandler et al., 2014; Plummer et al., 2015, 2017; Mulvey et al., 2018; Chen et al., 2019) and the explosion in techniques for genetic neuroanatomy are likely to identify more genes that are differentially expressed across LC modules, such as axon guidance molecules, neurotransmitter receptors, and cotransmitter peptides. Ongoing studies of gene expression at the single-cell level must be extended throughout development, and combined with LC projection tracing, for a clear and complete picture of this complex system.

Afferent circuitry may constrain LC efferent modules

Extensive exploration of how afferents integrate with LC microcircuits to activate specific, efferent-defined LC modules is necessary for understanding how the LC functions as a collection of modules. The inputs to the LC are not as globally diffuse as its efferent network, but it does receive input from >100 brain regions assessed in mice (Schwarz et al., 2015) and from a broad array of regions assessed in separate tracing studies in rats and monkeys (Aston-Jones et al., 1991). To study the input–output

mapping in the LC, researchers recently developed a combinatorial viral-genetic tracing tool called TRIO, which allows one to perform trans-synaptic rabies tracing from subsets of neurons defined by cell type and projection pattern (Schwarz et al., 2015). This work revealed that a majority of LC neurons receive similar input regardless of their projection target. On the other hand, recent work has provided an example of separate LC afferents evoking different LC-related behaviors (Yackle et al., 2017). While it has long been known that the LC response to severe stressors is driven by afferents from the paragigantocellular nucleus and the nucleus prepositus hypoglossi (Aston-Jones et al., 1986, 1991), recent work using TRIO-based viruses has demonstrated that the mild arousal evoked by a novel environment appears to be driven exclusively by *Cdh9/Dbx1* neurons in the breathing-related pre-Bötzinger complex (Yackle et al., 2017). Thus, different behavioral contexts may activate specific LC afferents; yet it is unknown how specific inputs affect the activity of subpopulations of LC neurons and the corresponding LC target-specific output.

In terms of neurochemical-selective manipulation of inputs to the LC, most work has focused on neuropeptide systems. Here, building on a strong background of anatomical and neurochemical studies, multiple groups have shown diverging functions of hypothalamic and amygdalar projections to the LC (Horvath et al., 1999; Reyes et al., 2006, 2008, 2011; Kravets et al., 2015). Hypothalamic hypocretin projections appear to modulate both arousal (Carter et al., 2012) and fear learning (Sears et al., 2013), whereas specific CeA input to the LC appears to robustly drive anxiety-like and aversive behaviors (McCall et al., 2015; Reyes et al., 2015) via stress-related corticotropin releasing hormone (Curtis et al., 1997, 2012; Lechner et al., 1997; Jedema and Grace, 2004; Devilbiss et al., 2012; Prouty et al., 2017). Corticotropin releasing hormone release in the LC dose-dependently alters glutamate responsivity of LC neurons (Prouty et al., 2017). Importantly, this potentially enables corticotropin releasing hormone to "tune" glutamatergic afferents in a way that may activate LC modules with function-specific efferents. The LC receives multiple glutamatergic afferents (from paragigantocellularis nucleus, lateral habenula, and PFC) (Herkenham and Nauta, 1979; Arnsten and Goldman-Rakic, 1984; Aston-Jones et al., 1986; Holloway et al., 2013). Activation of these afferents is associated with the onset of salient or goal-relevant stimuli (Aston-Jones et al., 1994; Bouret and Sara, 2004), as well as affective disorders, opiate withdrawal, and neuropathic pain (Aghajanian et al., 1994; Hayashida et al., 2010; Bernard et al., 2011; Chandley et al., 2014; Kimura et al., 2015). Elevated expression of NMDA ionotropic and metabotropic glutamate receptors in the LC have been observed in postmortem tissue from suicide victims, suggesting that such input—output-specific LC modules, activated by particular neurochemicals, may play a role in major depressive disorder (Bernard et al., 2011; Chandley et al., 2014). Similarly, recent evidence suggests that increased availability of metabotropic glutamate receptor 5 (mGluR5) is associated with suicide ideation in patients with post-traumatic stress disorder (Davis et al., 2019).

In light of these important implications for neuropsychiatric disorders, future studies will need to delve deeper into LC afferent pathways, their neurochemical identity, and how receptor subtypes are modularly organized within the LC. The emerging approach of defining LC modules by their receptor complement is exemplified by experiments demonstrating genetic differences underlying the higher glutamate-mediated excitability of LC neurons that project to the motor cortex (Chandler et al., 2014). Overall, it is clear that parsing the LC based on distinct projections, whether afferent or efferent, seems to hold some promise for identifying emergent properties of functional modules.

LC ensemble activity patterns permit targeted neuromodulation

Targeted neuromodulation by subpopulations of LC neurons could be achieved by LC neurons that do not spike synchronously (in addition to targets expressing different adrenergic receptors or differential densities of noradrenergic release sites). Longstanding evidence using single electrode recordings had suggested that LC neurons spike in population synchrony to achieve the global neuromodulation necessary for functions, such as wakefulness (Foote et al., 1980; Aston-Jones and Bloom, 1981). One study in the awake monkey had suggested that, among 23 pairs of LC neurons, levels of synchrony could fluctuate depending on cognitive state (Usher et al., 1999). Recent work using silicone probes with a high-channel density circumvented the limitation of single electrode recordings, allowing researchers to observe large-scale LC single-unit population activity for the first time (Totah et al., 2018, 2019). Importantly, by assessing >3000 cell pairs, this method revealed that an ensemble code does exist in the LC. Specifically, LC neurons were found to have overall little population synchrony. Moreover, the limited pairwise synchrony that existed occurred over specific timescales: submillisecond, tens of milliseconds, and infra-slow (multisecond) oscillatory synchrony. The authors speculate that these various timescales may relate, respectively, to gap junction connectivity, shared afferent inputs, and regulation of cortical networks formed by correlations of fMRI BOLD or mesoscale (e.g., local field potential) signals across brain regions. Totah et al. (2018, 2019) also used graph theoretic analyses to look beyond pairwise synchrony and demonstrate an LC ensemble code. Importantly, neurons with synchronous activity tended to project to functionally related forebrain regions; thus, an ensemble code combined with the efferent topography described above may permit a truly targeted neuromodulatory signal from the LC. Recordings of LC single-unit activity with high-channel-density electrophysiology in the awake, behaving animal is a crucial next step in understanding the signal conveyed by the modular architecture of the LC.

Charting a path forward by developing a multilevel understanding of the LC

A coherent and comprehensive picture of the LC will integrate multiple levels of observation across the noradrenergic system, including (1) the genotype and molecular/neurochemical phenotype of LC neurons, (2) their local interactions and ensemble activity patterns, and (3) the anatomy of their inputs and outputs and how these contribute to LC neuronal activity patterns in the context of various LC-associated functions. Such information can be used to inform and constrain existing and emerging models of noradrenergic neuromodulation that have not yet considered the implications of the modular LC architecture (Aston-Jones and Cohen, 2005a,b; Bouret and Sara, 2005; Yu and Dayan, 2005; Sales et al., 2019).

Such a path forward, with regard to genotype and molecular/neurochemical phenotype, is exemplified by recent work on LC galanin neurotransmission, stress, and anxiety (McCall et al., 2015), differences in gene expression depending on LC neuron projection target (Chandler et al., 2014), and new tools that combine genetic fate mapping and tracing of LC projections (Plummer et al., 2017). These methods reveal function-specific LC

modules that differ in projection target and genotype. However, LC neurons produce a multitude of neurochemicals and express a variety of genes (Mulvey et al., 2018). A better understanding of this level of diversity in the LC will allow the development and targeted deployment of research tools and pharmacological probes that can assess the functional roles of distinct LC neuronal subpopulations. This is likely to be of importance and utility in a wide range of disease states associated (at least partly) with the LC that range the developmental spectrum, such as attention-deficit and hyperactivity disorder, anxiety, depression, post-traumatic stress disorder, pain, Parkinson's disease, and dementia. Each of these neuropsychiatric disorders has plausible theories and demonstrable evidence of noradrenergic dysregulation, yet this manifests predominantly in particular domains, such as memory, movement, sensory processing, sleep disturbance, and emotional lability that may or may not be shared across these disorders. Treatments that can selectively target modular aspects of the LC noradrenergic system have a compelling logic that may enable a more effective amelioration of symptoms in specific domains with fewer side effects.

How many modules are contained within the several thousand neurons of the rodent LC (or indeed the estimated 60,000 of the primate/human brain) and how these modules can be engaged combinatorically or differentially remain to be determined. Understanding the intra-LC microcircuitry that allows the LC to activate modules independently (i.e., at different times) will require high-channel-density *in vivo* electrophysiology (Totah et al., 2018) to be combined with *in vitro* characterization of LC membrane properties and with optogenetic and chemogenetic activation of LC modules defined by their specific afferents and efferents using retrograde viruses and genetic fate mapping. Moreover, tools that identify brain states associated with activation of specific LC modules or enable profiling of discrete sites of transmitter release will be vital to resolving the functions of LC modules (Lovett-Barron et al., 2017; Feng et al., 2019; Zerbi et al., 2019).

Understanding of LC function will require an effort to integrate across levels, which necessitates collaboration among diverse scientists with different expertise, ideas, and perspectives. It is our hope, therefore, that new combinations of scientists and tools will be brought together to peer inside Reil's and the Wenzels' shimmering dark spot (Reil, 1809; Wenzel and Wenzel, 1812). If the nucleus, LC, is truly a collection of modules, then a return to the original nomenclature, the *Loci Caerulei* (Wenzel and Wenzel, 1812), *the many nuclei*, may be apropos.

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